1: Bone Marrow Transplant. 1992;9 Suppl 1:189-90. Related Articles, Links

Clinical application of intrauterine bone marrow transplantation for treatment of genetic diseases--feasibility studies.

Slavin S, Naparstek E, Ziegler M, Lewin A.

Dept. of BMT, Hadassah University Hospital, Jerusalem, Israel.

Intrauterine bone marrow transplantation (BMT) may represent a new approach for correction of a large variety of genetic disorders in utero. The procedure may become feasible for more genetic disorders in the future, since a large majority of potentially correctible diseases can be diagnosed at an early stage of gestation in utero using molecular probes that permit analysis of small biologic samples and even few cells that may be obtained by chorionic villi biopsy and/or amniocentesis. Haploidentical paternal marrow (2 cases) and sibling bone marrow cells from a disease-free family members, were infused into the fetus. GVHD was avoided following in vitro T-lymphocyte depletion using monoclonal antilymphocyte (CDW52) antibodies (Campath-1) without affecting stem cell viability, similarly to the procedures in routine use in clinical BMT programs in man. Three women underwent intrauterine BMT at 34, 23 and 25 weeks of gestation for metachromatic leucodystrophy (Arylsulfatase A deficiency, 2 cases) and beta thalassemia major (1 case), respectively. A total of 33 x 10(8), 30 x 10 (8) and 30 x 10(8) bone marrow cells were infused intraperitoneally (1 case), intraportally plus intraperitoneally (2 cases) with no fetal distress. Although the procedure was uneventful and no clinical evidence of GVHD was observed following delivery, correction of the basic disorders was not accomplished because of anticipated rejection of marrow allografts. (ABSTRACT TRUNCATED AT 250 WORDS)

PMID: 1504665 [PubMed - indexed for MEDLINE]

1: Fetal Diagn Ther. 1990;5(1):40-56. Related Articles, Links

New approaches in bone marrow transplantation: the utilization of hematopoietic stem cells.

Michejda M, Peters S, De Vleeschouwer MH, Bellanti JA.

International Center for Interdisciplinary Studies of Immunology, Georgetown University School of Medicine, Washington, D.C.

A detailed review of various methods of bone marrow transplantation is presented. Special emphasis is placed on the newest reports of fetal bone marrow transplantation in utero and stem cell reconstitution.

Publication Types:
- Review
- Review, Tutorial

PMID: 1983166 [PubMed - indexed for MEDLINE]

1: Am J Pediatr Hematol Oncol. 1994 Feb;16(1):35-42.

In utero hematopoietic stem cell transplants for inherited diseases.

Cowan MJ, Golbus M.

Department of Pediatrics, University of California, San Francisco 94143-1278.

PURPOSE: The treatment of choice for many inherited diseases is bone marrow transplantation (BMT). Limitations to using marrow transplants for inherited diseases include (a) the toxicity associated with high doses of chemotherapy necessary to obtain engraftment; (b) the complications associated with graft-versus-host disease (GVHD); (c) the fact that only 20-25% of children will have a human leukocyte antigen (HLA)-matched donor; and (d) the concern that, at least for some inherited diseases, significant organ damage, especially to the nervous system, has occurred by the time the child is diagnosed and evaluated for possible BMT. In utero transplantation of hematopoietic stem cells (HSCs) offers the possibility of overcoming many of these limitations. PATIENTS AND METHODS: One of the biggest hurdles to a successful transplant is the ability of the recipient to reject the donor marrow. Except in patients with severe combined immunodeficiency disease (SCID), overcoming this hurdle requires high doses of chemotherapy. Early in gestation, the fetus is significantly immunoincompetent. Before 14-15 weeks of gestation, the human fetus appears to be similar to a child with SCID in its inability to reject allogeneic cells. Potential sources for HSCs are HLA-matched sibling marrow, fetal liver, parental bone marrow, and cord blood. RESULTS: With fetal liver, only cells from fetuses < 10-12 weeks are acceptable because of the high risk of GVHD. With parental marrow, the cells must be T cell depleted in order to minimize the risk for GVHD. Problems in using fetal liver include the inability to obtain sufficient numbers of cells and inadequate supplies of donor tissue. The source and supply of parental bone marrow is almost unlimited, but, because of the need for T-cell depletion, bone marrow from a parent may have a lower engraftment rate in the child. CONCLUSIONS: Studies in fetal murine and Rhesus models using fetal liver or T cell-depleted bone marrow from adult animals suggest that engraftment can be

successfully obtained, providing the transplant is performed sufficiently early in gestation. To date, at least a dozen in utero human transplants have been attempted worldwide in fetuses diagnosed with a variety of inherited diseases. Because of the small number of transplanted fetuses and the variety of diseases and differing transplant conditions, it is difficult to draw any firm conclusions regarding ultimate efficacy of the procedure and its risk. However, it does appear that the age of gestation of the recipient, the dose of cells infused, and possibly the route of administration of the HSCs will be critical factors in determining success rates for this approach. The successful application of in utero transplantation would allow treatment of a variety of inherited diseases early in gestation while eliminating many of the risks associated with conventional BMT.

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Santner-Nanan B, Aust N Z J Obstet Gynaecol. 2005 Apr;45(2):102-7. Therapeutic potential of stem cells in perinatal medicine.

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Article identifier	1079979601000894
Authors	Mackenzie_T_C Flake_A_W
Journal title	Blood Cells, Molecules, and Diseases
ISSN	1079-9796
Publisher	Academic Press USA
Year of publication	2001
Volume	27
Issue	3
Supplement	0
Page range	601-604
Number of pages	4
User name	Adonis
Cost centre	
PCC	$35.00
Date and time	Wednesday, September 07, 2005 11:56:11 PM

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doi:10.1006/bcmd.2001.0424, available online at http://www.idealibrary.com on IDEAL®

Human Mesenchymal Stem Cells Persist, Demonstrate Site-Specific Multipotential Differentiation, and Are Present in Sites of Wound Healing and Tissue Regeneration after Transplantation into Fetal Sheep

Tippi C. Mackenzie[1] and Alan W. Flake[1]

Submitted 05/08/01
(Communicated by M. Lichtman, M.D., 05/08/01)

ABSTRACT: Prenatal transplantation of stem cells is an exciting frontier for the treatment of many congenital diseases. The fetus may be an ideal recipient for stem cells, as it is immunologically immature and has rapidly proliferating cellular compartments that may support the engraftment of transplanted cells. Mesenchymal stem cells (MSC), given their ability to differentiate into multiple cell types, could potentially be used to treat diseases such as osteogenesis imperfecta, muscular dystrophy, and other mesenchymal disorders that can be diagnosed *in utero*. We have shown, using a human–sheep *in utero* xenotransplantation model, that human MSC have the ability to engraft, undergo site-specific differentiation into multiple cell types, and survive for more than 1 year in fetal lamb recipients. In addition, in this model MSC-derived cells appear to be present in increased numbers in wounded or regenerating tissues. This observation warrants further studies of the biology of MSCs following systemic or site-directed transplantation.

INTRODUCTION

Mesenchymal stem cells (MSC) are multipotential stem cells that have been isolated from the bone marrow of many species, including humans (1). Given their potential to differentiate into tissues such as bone, cartilage, fat, and muscle (2, 3), they have the potential for cellular treatment of a variety of congenital and acquired diseases. An attractive feature of MSCs is the ability to isolate them from an adult bone marrow aspiration, expand them *ex vivo*, and utilize them for autologous tissue engineering or transplantation applications. However, for diseases in which a defective cell population requires replacement, transplantation of allogeneic MSC may still be required with the attendant morbidity of myeloablative conditioning or immunosuppression (4). In addition it remains unclear whether systemically administered MSC can home and engraft in tissues with clinically relevant efficiency.

Prenatal cellular transplantation may be an approach to circumvent barriers to allogeneic engraftment. Multiple studies with hematopoietic stem cells (HSC) have shown that the immunologically immature fetus, can accept allogeneic and xenogeneic grafts without prior marrow conditioning (5, 6). Furthermore, introduction of foreign cells with appropriate presentation of antigen to the thymus during thymic processing of self antigen may induce tolerance to the transplanted cells. Because of the small size of the fetus, and the normal proliferative and migrational events during ontogeny, the potential exists for engraftment of donor cells into a number of different stem cell compartments with subsequent extensive expansion. Finally, successful prenatal engraftment may preempt clinical manifestations of a disease, a consideration that is important in disease phenotypes in which damage begins before or soon after birth. Despite these advantages,

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clinical success has only been achieved when there is a survival advantage for the engrafted cells, such as in SCID (7, 8). Congenital musculoskeletal diseases such as osteogenesis imperfecta (9) and muscular dystrophy (10, 11) provide a "donor cell advantage" and could possibly be treated by strategy of *in utero* MSC transplantation. An additional application is to utilize MSCs to facilitate engraftment of HSCs following pre- (12, 13) or postnatal HSC transplantation (14).

MATERIALS AND METHODS

The Human–Sheep Model

To investigate the transplantation biology, differentiation potential, and potential utility of MSCs for *in utero* transplantation we modified an animal model that has been very useful for studies in prenatal human HSC transplantation; the xenogeneic human–sheep model. Originally developed as an assay system for human hematopoiesis, this model has been utilized to assay a variety of human hematopoietic cell populations for long-term repopulating capacity (15, 16). The xenogeneic system allows the identification of engrafted human cells within the sheep background using a variety of human specific methodologies such as PCR, FACS, immunohistochemistry, and *in situ* hybridization. Since the early gestational fetal lamb is tolerant to xenogeneic cells, we hypothesized that this would be an ideal system in which to study the behavior of human MSC following systemic administration. To conduct these studies we chose a relatively defined population of human MSC (2) which have been shown *in vitro* to differentiate into bone, cartilage, and adipose tissue. In our initial study, these cells were transplanted by intraperitoneal injection (5 to 20 $\times$ 10^6 cells or approximately 1 $\times$ 10^8 cells/kg) into early (65 days; term = 145 days) and mid (85 days) gestational fetal lamb recipients (representing pre and post immunocompetence developmental time points). The mid gestational time point was used in order to test whether MSC have an immunomodulatory role *in vivo* as the 85 day gestation lamb is capable of rejection of allo or xeno skin or cellular grafts. At multiple time points following transplantation, the animals were sacrificed and their tissues were analyzed for evidence of human cell engraftment.

In a second study, we have performed identical transplants at 65 days gestation and performed a second fetal procedure at 100 days gestation consisting of wounding various tissues, including skin, muscle, cartilage, tendon, and bone, or, a partial hepatectomy. These studies were designed to determine if engrafted MSCs would proliferate in response to tissue injury, and perhaps participate in repair or regeneration. The wounded areas were analyzed at various time points and compared to a nonwounded contralateral control.

RESULTS AND DISCUSSION

In our initial study (17) 28/29 animals tested showed evidence of engraftment in one or more tissues by PCR. Immunohistochemistry for β-2 microglobulin (a component of class I MHC) was used to confirm human cell engraftment and confirmed the presence of human cells in cartilage, fat, skeletal and cardiac muscle, lung, thymus, spleen, and brain. We observed that almost all tissues contained evidence of human cells at early time points, whereas there was a variable pattern of engraftment at the later time points. Overall, MSC persisted for more than 13 months, even when transplanted into immunocompetent fetuses.

To assess differentiation of engrafted cells, we used a combination of immunohistochemistry, *in situ* hybridization, and cell morphology to identify specific cell types. In cartilage, we observed cells staining for β-2 microglobulin within cartilage lacunae, indicating chondrocytic differentiation. In the thymus, we observed cells staining for human CD74, a marker for thymic epithelial cells (Fig. 1d). In bone marrow, cells staining for human CD23 confirmed differentiation into bone marrow stromal cells (Fig. 1b). (Although CD23 is also found on B cell progenitors, the injected mixture did not contain any hematopoietic precursors and CD45 staining of bone marrow from these chimeras has been negative.) Double staining with β-2 microglobulin and various markers of myogenic differentiation such as

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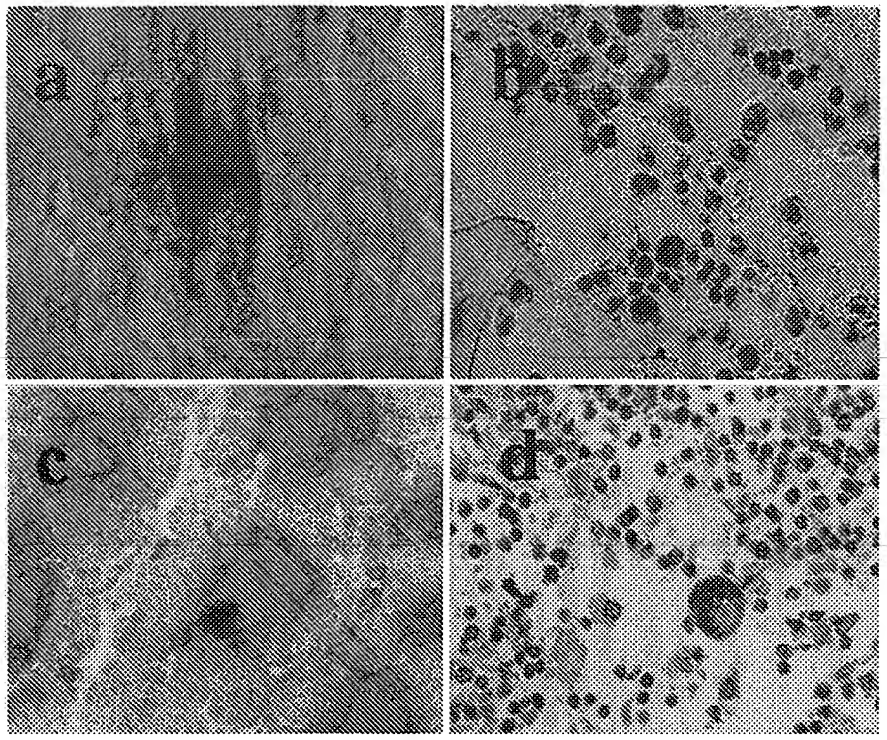

FIG. 1. Immunohistochemistry on chimeric sheep tissues demonstrating the presence of engrafted human cells with site-specific differentiation. (a) Chimeric cardiac muscle double stained with SERCA-2 (pink) and β-2 microglobulin (brown) demonstrating one human-derived cardiomyocyte. (b) Chimeric bone marrow stained with human-specific CD23, demonstrating multiple human-derived marrow stromal cells. (c) Chimeric skeletal muscle stained with myosin heavy chain (slow type, red) and β-2 microglobulin (black), demonstrating one human-derived skeletal myocyte. (d) Chimeric thymus stained with human-specific CD74 (brown), demonstrating one human-derived thymic epithelial cell.

SERCA-2, myosin, and dystrophin indicated differentiation into skeletal muscle. (Fig. 1c) This observation was confirmed with colocalization of myogenic markers in cells with human nuclei in subsequent *in situ* hybridization experiments, in which probes for human specific alu sequences were used. Finally, in cardiac muscle, staining with SERCA-2 and either β-2 microglobulin (Fig. 1a) or *in situ* hybridization showed cardiomyocytes of human origin.

These findings warrant further testing of the capacity of different populations of MSC to differentiate along multiple lineages following systemic transplantation. It is possible that a more primitive population of MSC may demonstrate better engraftment or more widespread differentiation than the population of cells used in this study. Although we did not observe differentiation into neurons or astrocytes (18) in this study, it is possible that such a capacity may be realized in a nonxenogeneic setting.

Tissue injury may induce homing of these cells to particular tissues with subsequent differentiation in response to the local tissue microenvironment, as has been seen in models of hepatocytic differentiation of HSC (19, 20). Alternatively, engrafted cells in the local environment may be induced to proliferate and participate in the repair or regenerative response. We have seen evidence of this in preliminary analysis of wounds inflicted after *in utero* MSC transplantation in our model. When compared with contralateral controls, a relatively large number of human cells can be seen within the area of the wound.

Since clinical applications of MSC transplantation will require delivery of a larger number of cells to a particular target, such as muscle or cartilage, the behavior of these cells following direct injection to a particular site (survival, differentiation, and systemic spread) needs to be investigated. Although this approach would not be expected to induce tolerance by classic mechanisms of thymic deletion, our observation that

MSC persist even after transplantation into immunocompetent fetuses suggest an immunomodulatory role for these cells (21). Finally, transplantation of genetically modified MSC, both in this model and in a disease model, may answer questions regarding longevity of transgene expression *in vivo*.

ACKNOWLEDGMENTS

This paper is based on a presentation made at the Focused Workshop on Stem Cell Plasticity sponsored by The Leukemia & Lymphoma Society and the Great Basin Foundation for Biological Research in Santa Barbara, California, on May 4 and 5, 2001.

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Engraftment of Bone Marrow and Fetal Liver Cells After In Utero Transplantation in MDX Mice

By Tippi C. MacKenzie, Aimen F. Shaaban, Antoneta Radu, and Alan W. Flake
Philadelphia, Pennsylvania

Background/Purpose: In utero hematopoietic stem cell transplantation (IUHSCTx) has been experimentally or clinically effective only in circumstances in which there is a survival advantage for donor cells. A survival advantage exists for normal muscle cells in muscular dystrophy. Because hematopoietic and mesenchymal stem cells may have the capacity to differentiate into muscle cells, the authors hypothesized that in utero bone marrow (BM) or fetal liver (FL) stem cell transplantation may be used to treat muscular dystrophy.

Methods: Time-dated 14-day-gestation fetal muscular dystrophy mice (*mdx*) were injected intraperitoneally with 1 to 5 × 10^6 BM or FL cells per fetus from Rosa26 donor mice (transgenic for lacZ). Four weeks after birth, peripheral blood from the pups was analyzed for hematopoietic chimerism by using fluorescence-activated cell sorting (FACS) for the Ly-9.1 marker. Chimeric mice (6 BM and 2 FL recipients) were sacrificed at 12 to 14 months of age, muscles were stained with X-gal, and analyzed by 1- to 2-μm plastic sections. Polymerase chain reaction (PCR) for lacZ was performed in other organs to determine systemic engraftment.

Results: At the time of death, all animals that were chimeric at 4 weeks continued to show hematopoietic chimerism of 0.2% to 9% by FACS. Engrafted donor cells were found in multiple sections from hindlimb skeletal muscles, diaphragms, and hearts from both BM and FL recipients. These cells had incorporated into the host muscles, and their morphology was consistent with myogenic differentiation. PCR of BM, liver, spleen, thymus, kidney, and lung for lacZ was positive in multiple animals.

Conclusions: IUHSCTx leads to widespread engraftment of donor cells in multiple muscle compartments of hematopoietic chimeras. The advantage for normal myocytes offered in the *mdx* model allows engraftment and myogenic differentiation of transplanted BM or FL cells by morphology at a relatively higher frequency in muscle relative to other tissues, without the need for host conditioning. Because muscular dystrophy now can be detected early in gestation, such a strategy may offer a future alternative in the clinical treatment of this disease.

INDEX WORDS: Muscular dystrophy, *mdx* mice, in utero transplantation, stem cell engraftment.

IN UTERO HEMATOPOIETIC stem cell transplantation (IUHSCTx) may be used theoretically to correct a wide variety of congenital diseases by replacing a defective cell lineage with functional cells.[1] The potential advantages of IUHSCTx over postnatal therapy include the naïve fetal immune system (allowing tolerance for allogeneic cells), the remarkably proliferative environment of the early gestational fetus, and the normal ontologic migrations of stem cells to multiple tissue compartments. Prenatally transplanted allogeneic stem cells can therefore engraft, expand, and migrate to seed various tissues without the need for immunosuppression or myeloablation. However, there are barriers limiting engraftment and expansion in the fetus: experimental and clinical successes thus far have been limited to diseases in which there is a clear survival advantage for donor cells, such as severe combined immunodeficiency.[2,3]

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease that causes muscle degeneration early in childhood. Affected individuals suffer from cardiomyopathy, and death ensues generally from respiratory failure.[4] The genetic defect has been localized to the dystrophin gene, encoding a 427-kD membrane protein, which is believed to stabilize the cell membrane during muscle contractions.[5] A mutation in dystrophin leads to a lowered threshold of injury, which results in damage to the muscle fibers during contractions.[6] The mouse model for muscular dystrophy, the *mdx* mouse,[7] has a point mutation in dystrophin[8] and undergoes repeated cycles of degeneration and regeneration during its lifetime. Al-

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Presented at the 53rd Annual Meeting of the Section on Surgery of the American Academy of Pediatrics, San Francisco, California, October 19-21, 2001.

This work was supported in part by National Institutes of Health Grants NRSA F32 HL10229-01 (T.C.M), R01 HL/DK63434 (A.W.F), RO1 HL64715 (A.W.F), an American College of Surgeons Resident Research Scholarship (T.C.M), a grant from the Muscular Dystrophy Association (A.W.F), and funds from the Ruth and Tristram C. Colket, Jr, Chair of Pediatric Surgery.

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0022-3468/02/3707-0025$35.00/0
doi:10.1053/jpsu.2002.33844

though initially muscle stem cells (satellite cells) replicate to repair and regenerate the injured muscle, over the course of the life of the animal, these cells are depleted gradually;[9] and the mice show progressive weakness and muscle deterioration.[10]

Current treatment approaches to DMD involve transplantation of myoblasts from an HLA-compatible donor, but their survival in the affected muscle has been extremely limited.[11,12] The primary problems with this approach are the immune response and limited distribution of donor cells to the large mass of muscle tissue affected by the disease. Because muscular dystrophy can be detected in utero,[13,14] the advantages of the fetal environment might address the current limitations of postnatal cellular therapy for this disease.

We have shown previously that transplantation of hematopoietic stem cells (HSC) from fully allogeneic bone marrow (BM) or fetal liver (FL) into 14-day-gestation fetal mice results in long-term multilineage hematopoietic chimerism.[15] These chimeras are fully tolerant to the donor strain after a single in utero stem cell transplant.[16,17] Evidence from postnatal HSC transplants has show that HSC can differentiate into muscle in cases of injury[18] or in the background of the *mdx* mice.[19] Mesenchymal stem cells (MSC), another stem cell population found in bone marrow,[20,21] also may be induced to differentiate into skeletal and cardiac muscle after in utero administration, as we have recently shown in a fetal lamb model.[22]

Because the preparations of BM and FL we use in the creation of hematopoietic chimeras contain both HSC and MSC, we hypothesized that IUHSCTx might be used to achieve muscle cell engraftment in addition to hematopoietic chimerism in the *mdx* mouse. We therefore injected *mdx* fetuses with BM or FL cells from donor mice that were transgenic for the marker gene *lacZ*, and examined the fate of these cells after 1 year in the recipients. We report here that in addition to long-term hematopoietic chimerism, donor cell engraftment in the heart and muscular compartments with myocytic differentiation as seen by morphology occurred.

MATERIALS AND METHODS

Animals

Mdx mice (C57BL10ScSn/DMD^{mdx})[7] and Rosa26 mice (B6, 129Sgtrosa26)[23] were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at our animal facility at the Abramson Pediatric Research Center. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and followed guidelines set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Bone Marrow and Fetal Liver Cell Harvests

Harvest and in utero transplantation of BM or FL was performed as previously described.[24] Briefly, bone marrow harvest was performed by flushing the tibias and femurs from 2- to 3-month-old donor Rosa26 mice with phosphate-buffered saline (PBS). A single-cell suspension was created by filtering through a 75-μm filter, and the mononuclear layer was separated on a Ficoll density gradient (Histopaque 1077; Sigma, St Louis, MO). The mononuclear layer was collected, washed with sterile PBS (Gibco) twice, and counted. Fetal liver was collected from 14-day-gestation Rosa26 fetuses, which were harvested from anesthetized pregnant dams. The fetal liver was harvested by dissection, gently homogenized by multiple passages through a 1-mL syringe, and filtered through a 75-μm filter. The mononuclear layer was separated on a Ficoll density gradient, and the cells were washed and counted.

In Utero Stem Cell Injections

Time-dated pregnant *mdx* dams at 14 days' gestation were anesthetized using metophane, and a midline abdominal incision was made to expose the uterus. The fetuses were injected intraperitoneally using a 100-μm beveled pulled glass micropipette through the uterine wall, which is translucent at this gestational age. Each fetus was injected with 5 μL of either bone marrow (5×10^6 cells per fetus) or fetal liver (1×10^6 cells per fetus). The abdomen was closed in 2 layers with 5-0 absorbable suture, and the mice were returned to their cages.

FACS Analysis of Hematopoietic Chimerism

Blood was drawn at 4 weeks and at the time of sacrifice using retroorbital puncture. Mononuclear cells were isolated using a Ficoll density gradient and stained with directly conjugated anti Ly-9.1-FITC and anti CD45-PE antibodies (Pharmingen, San Diego, CA), Propidium iodide staining was used to exclude dead cells from the analysis. The cells were counted by 2-color flow cytometry (FACScan, Becton Dickinson, San Jose, CA). The percent donor cells was defined as (Ly-9.1+ and CD45+ cells)/ all CD45+ cells.

Mdx mice are Ly-9.2, whereas Rosa mice are carriers for Ly-9.1. Because Rosa26 transgenic mice were created by injection of pseudopregnant C57 mice (Ly-9.2) with embryonic stem cell lines from 129Sv mice (Ly-9.1), which then were back-bred with 129Sv mice,[23] the Ly-9.1 allele is expressed in some animals but is lost in others (range 0 to 100%). Therefore, the percentages of chimerism obtained by FACS for Ly-9.1 significantly underestimate the percentage of cells that are from Rosa26. At least 8 Rosa26 donor animals were used to prepare the cell populations for BM or FL transplants to allow a sufficient sample of Ly-9.1-expressing donors.

Polymerase Chain Reaction Analysis

Genomic DNA was isolated from tissues (which were snap-frozen in liquid nitrogen at the time of harvest) using DNAzol (Molecular Research Center, Cincinnati, OH), or MasterPure Genomic DNA isolation kit (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions. PCR for lacZ was performed using the primers upper 5'-CGTCACGAGCATCATCCT-3' and lower 5'-TCCGCCGCCTTCATACTG-3', using 1 μg of DNA per reaction. The reaction conditions were as follows: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by 5 minutes at 72°C. PCR also was performed for the housekeeping gene β-actin as an internal control, using the primers upper 5'-CGGGACCTGACTGACTAC-3' and lower 5'-GAAGGAAGGCTGGAAGAG-3' with the same reaction conditions as above except that 35 cycles were performed at 55°C. PCR products were visualized on a 3% agarose gel.

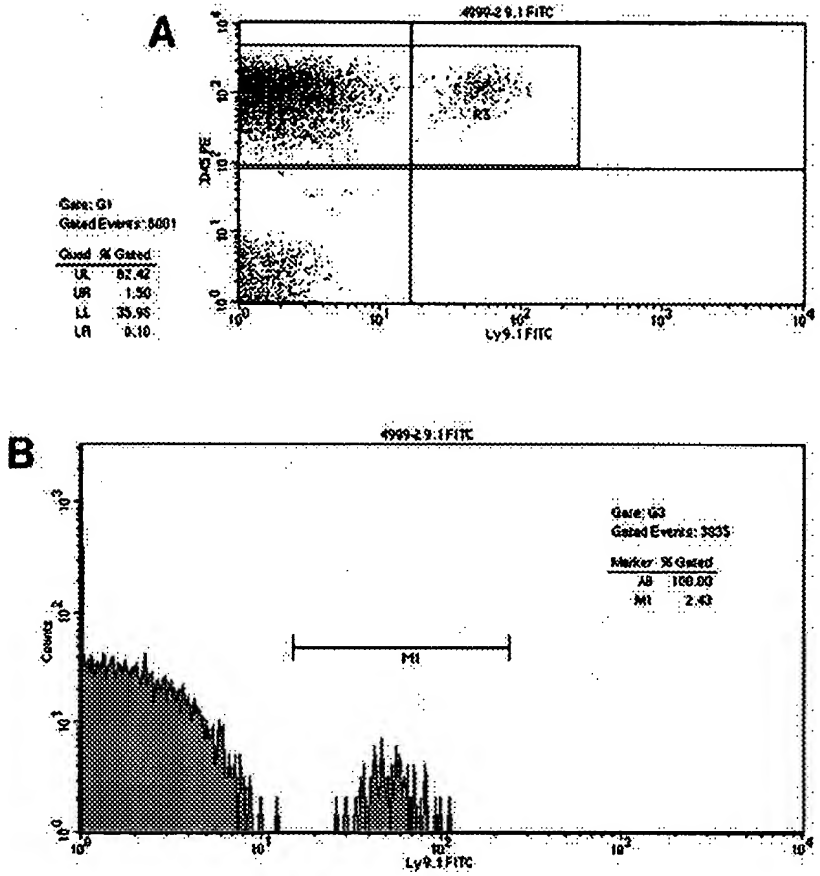

Fig 1. (A) Dual color flow cytometric scatter plot of peripheral blood mononuclear cells from a hematopoietic chimera stained with anti-Ly9.1-FITC and anti-CD45-PE antibodies. Donor-derived hematopoietic cells are both Ly9.1 and CD45 positive and appear in the right upper quadrant. (B) Histogram plot shows the amount of chimerism as the number of Ly 9.1 and CD45+ cells over all gated CD45+ cells.

Immunohistochemistry With Frozen Sections

At the time of death, tissues for analysis were frozen in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) in a dry ice/isopentane bath. Cryosections of 5 μm were cut, fixed with 0.5% glutaraldehyde, and X-gal stained with a solution containing 1 mg/mL of 5-bromo-4-chloro-3-indoyl-β-D-galactopyronidase, 5 mmol/L $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, and 1 mmol/L $MgCl_2$ in PBS, pH 7.4, overnight at 37°C. They then were counterstained with 0.5% neutral red, dehydrated over an alcohol gradient to xylene, and coverslipped.

Histochemistry With Plastic Sections

For plastic sections, tissues were cut into 2- to 3-mm pieces immediately after harvesting, fixed in 0.5% glutaraldehyde for 15 minutes, and X-gal stained overnight at 37°C as described above. They were postfixed with 10% neutral buffered formalin (Sigma) overnight, then dehydrated over an alcohol gradient to JB-40 infiltration medium (composed of JB-4 Plus solution (Electron Microscopy Sciences, Ft Warringon, PA) and 1% JB-4 Plus catalyst (Polysciences, Inc, Warrington, PA)). Tissues were placed in embedding molds using a 15:1 mixture of infiltration medium and JB-4 Plus accelerator (Polysciences, Inc), capped tightly with block holders, and incubated overnight at 4°C until polymerization was complete. Plastic sections of 2 μm were obtained on a Microm-HM 355 microtome, counterstained with neutral red or H&E, and coverslipped. Because the muscles in the *mdx* mouse undergo multiple cycles of degeneration and regeneration with secondary inflammatory infiltrates, age-matched *mdx* negative controls were used in the analysis to avoid significant differences in the inflammatory process in muscles.

RESULTS

IUHSCTx Leads to Long-Term Hematopoietic Chimerism

Four weeks after birth, peripheral blood from the transplanted surviving pups was analyzed for hematopoietic chimerism by using FACS for Ly9.1 and CD45 and by PCR for lacZ. In the FACS plot in Fig 1A, donor hematopoietic cells express both Ly 9.1 and CD45 (the common leukocyte antigen) and can be found as a separate population in the right upper quadrant. Figure 1B is a histogram that shows the percent of CD45 cells that are Ly 9.1 (donor derived). PCR for lacZ was performed to confirm chimerism: all animals that were positive by FACS were also PCR positive (data not shown). At the time of sacrifice, FACS again was performed on blood and BM of animals to determine if hematopoietic chimerism was persistent. Although initial hematopoietic chimerism was 0.4–3% of CD45 cells by FACS, at the time of death, animals continued to show

Table 1. Levels of Chimerism and Correlation With Muscle Histology

Animal No.	Donor Cell Type	4-week Chimerism (%)	1-Year Chimerism (%)	Muscle Histology
1	BM	PCR only	0.2	Negative
2	BM	2.4	4	Positive diaphragm, heart
3	BM	0.5	0.4	Negative
4	BM	2.77	2.1	Positive muscle
5	BM	1.28	0.9	Positive diaphragm
6	BM	1.05	0.85	Positive heart, muscle
7	FL	2	9	Positive muscle
8	FL	1.9	0.2	Negative

hematopoietic chimerism of 0.2% to 9% by FACS (Table 1). Hematopoietic chimerism was linked to evidence of donor cells in muscles by histology, with no cells observed in chimeras that were less than 0.8% by FACS, even if there was PCR evidence of chimerism in the blood.

Engraftment of Donor Cells in Muscles

Chimeric mice (6 BM and 2 FL recipients) were sacrificed at 12 to 14 months of age to allow adequate time for donor myogenic differentiation and replacement of degenerating muscle. Muscles (hindlimbs, diaphragms, and hearts) were stained with X-gal and analyzed by high-resolution 1 to 2-μm plastic sections. Blue cells, indicating engrafted donor cells, were found in multiple sections from hindlimb skeletal muscles, diaphragms, and hearts from both BM and FL recipients (Fig 2). These cells had incorporated into the host muscles. Two groups of cells were identified: the first population consisted of small cells that appeared to be donor-derived hematopoietic cells participating in the normal *mdx* inflammatory process (Fig 2 A and 2 B). This was most evident in the diaphragm, which begins to show signs of degeneration earliest because of its constant workload.[25] Small cells also were found incorporated into skeletal muscle architecture (Fig 2 C-E). The second population, more rare, appeared to be myocytes by morphology, with elongated fibers showing punctate

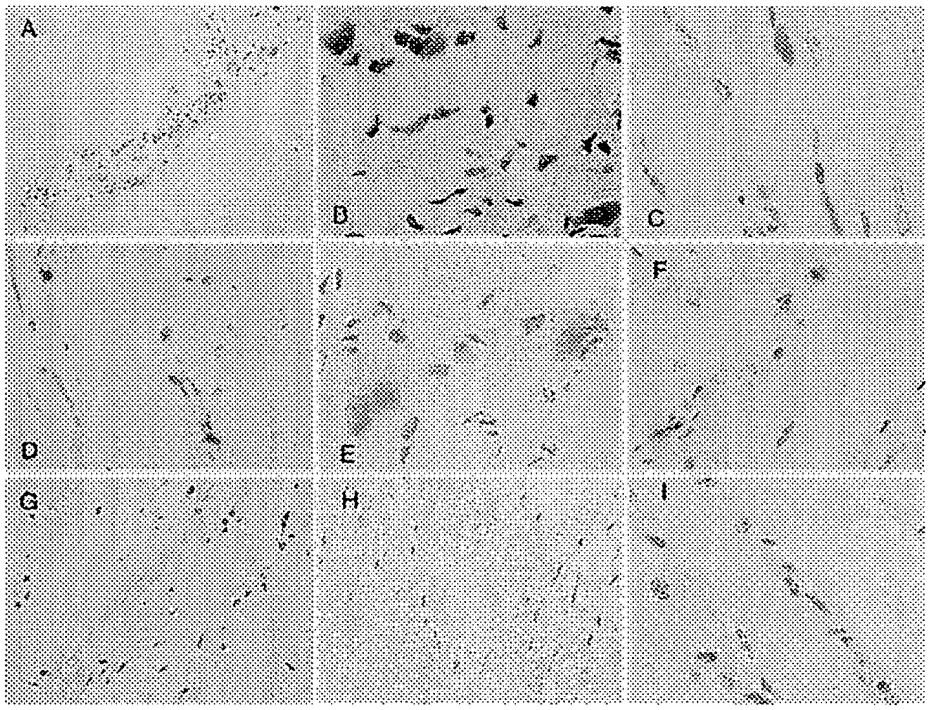

Fig 2. Plastic sections of X-gal stained muscles from chimeric (A-G) or control (H-I) mice. (A) Diaphragm, eosin counterstain, original magnification ×20. (B) Diaphragm, H&E counterstain, original magnification ×100. (C) Tibialis anterior, eosin counterstain, original magnification ×100. (D) Gluteus maximus, H&E counterstain, original magnification ×40. (E) Diaphragm, eosin counterstain, original magnification ×100. (F) Gluteus maximus, H&E counterstain, original magnification ×40. (G) Heart, eosin counterstain, original magnification ×40. (H) Rosa26 heart, eosin counterstain, original magnification ×40. (I) *Mdx* diaphragm, eosin counterstain, original magnification ×40.

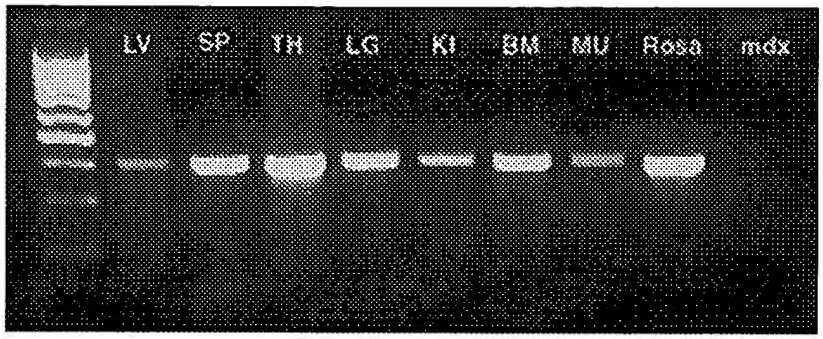

Fig 3. PCR gel of hematopoietic and nonhematopoietic tissues from one animal shows bands representative of the lacZ PCR product. Load order, molecular size markers; LV, liver; SP, spleen; TH, thymus; LG, lung; KD, kidney; MU, muscle, Rosa control, *mdx*-negative control. PCR for the housekeeping gene β-actin indicated the presence of DNA in all samples (data not shown).

lacZ staining and peripheral nuclei (Fig 2 F-G). These cells are a more faint blue than seen in Rosa controls, likely because muscle fibers are multinucleated, and the β-galactosidase enzyme that is produced in the donor-derived nucleus is "diluted" over a large muscle fiber. Rosa control mice also showed a punctate pattern and variable intensity of staining (Fig 2H). No such blue cells were found in age-matched *mdx* negative controls (Fig 2I).

Engraftment of Donor Cells in Other Organs

To determine if hematopoietic chimerism leads to engraftment of donor cells in other tissues, PCR for the lacZ gene was performed on hematopoietic and nonhematopoietic tissues. PCR of BM, liver, spleen, thymus, kidney, and lung for lacZ was positive in multiple animals (Fig 3). However, preliminary lacZ staining of frozen sections from these tissues has not shown donor cells on histology.

DISCUSSION

We have shown that IUHSCTx results in hematopoietic chimerism in this semiallogeneic strain combination. Both adult BM and FL transplants give rise to long-term chimerism and engraftment of donor cells in muscles. The in utero transplantation approach takes advantage of the normal ontogeny of the fetal immune system, permitting engraftment with donor-specific tolerance, and avoiding the need for myeloablation or immunosuppression.

The mouse model for DMD, the *mdx* mouse, has a less severe phenotype than seen in human DMD.[26] Mice have a normal life span but do show histologic evidence of muscle damage (such as central nuclei and evans blue uptake) during their lifetime, which can be corrected experimentally by expression of dystrophin in the muscles.[27] The changes start first in the diaphragm[25] and are evident in skeletal muscles later in life. It would be expected, therefore, that wild type cells with functional dystrophin would have a survival advantage over the host cells as the animal ages. The late time-point of analysis was chosen to allow sufficient time for engraftment, at a time when the resident satellite cell pool of the muscles would be expected to decline. Although some of the blue cells in the muscles appear to be inflammatory cells derived from the donor hematopoietic chimerism, the donor cell advantage offered in the *mdx* model also allowed detectable myogenic differentiation of engrafted stem cells by morphology. Because the muscle degeneration observed in the *mdx* model occurs more slowly and is less extensive than observed in human DMD, we would expect the selective advantage for engrafted normal cells in human DMD to be greater than that shown in this study, with presumably more rapid kinetics. Future experiments with more severe mouse models of muscular dystrophy[28,29] will address this hypothesis.

The observation that hematopoietic chimerism leads to PCR positivity in other tissues is interesting in light of recent discoveries that bone marrow–derived cells may be stem cells for tissues derived from other germ layers, such as liver[30,31] and brain.[32] Given the uniquely proliferative environment of the fetus and the strong environmental cues to promote differentiation of transplanted cells, it would not be surprising if similar stem cell plasticity could be seen in a fetal model. However, our positive PCR results in BM, liver, spleen, thymus, lung, and kidney may be secondary to contaminating hematopoietic elements. Alternatively, the differentiated donor cells in tissues other than muscle may be too low in frequency to detect by our preliminary histology. We interpret the finding of higher numbers of donor cells in muscles compared with other tissues to indicate a survival advantage of cells in muscles. Donor-derived hematopoietic cells also may be recruited more efficiently to muscles during the *mdx* mouse's regeneration process.

We chose to perform plastic sections for histologic analysis to improve resolution so that muscle differentiation could be documented by morphologic criteria. Another method of proving myogenic differentiation is to perform immunohistochemistry for dystrophin. Be-

cause *mdx* muscles are expected to be negative for dystrophin, positive fibers should be donor derived. However, because the *mdx* mouse has only a point mutation in the dystrophin gene, there is a well-documented high frequency of spontaneous revertants in these mice.[33] Because our level of engraftment is low, it would be difficult to distinguish dystrophin-positive cells that are donor-derived from revertant recipient cells. Future experiments that combine the in utero approach with a postnatal boosting should improve levels of engraftment and allow definitive demonstration of myogenic differentiation by dystrophin staining.

These results support the potential advantages of IUHSCTx for the treatment of DMD. The engraftment and persistence of minor-mismatch allogeneic cells for over 1 year as hematopoietic chimerism and cardiac and skeletal myocytes by morphology support the presence of immunologic tolerance. The wide distribution of the cells to multiple muscle compartments supports the migration and seeding of tissue compartments by stem cells transplanted in utero. If higher levels of engraftment can be achieved, these advantages could result in replacement of degenerating muscles as it occurs, resulting in clinical amelioration of DMD. Currently, we are developing methods to dramatically increase chimerism by a combined approach of IUHSCTx for prenatal tolerance induction with enhancement of mixed chimerism after birth by nonmyeloablative transplant regimens.

Diseases most amenable to prenatal treatment by stem cell transplantation should be detectable in utero, offer a survival advantage for donor-derived cells, and lack equally successful treatment options postnatally. Muscular dystrophy can be diagnosed by in utero muscle biopsy[14] or genetic analysis. Wild-type muscles should have a survival advantage over those lacking dystrophin, as they can better withstand the stress of repeated contractions. Because postnatal myoblast transplantation approaches have met with limited or no success,[34] a prenatal transplantation strategy may offer an alternative for clinical treatment of this disease in the future.

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Immunological tolerance induced by in utero injection.

Barnes RD, **Pottinger BE**, **Marston J**, **Flecknell P**, **Ward RH**, **Kalter S**, **Heberling RL**.

Intrauterine injection of human whole blood into rabbit and rhesus monkey fetuses was found to result in long lasting unresponsiveness to human serum albumin. Intrauterine injection of viable allogeneic bone marrow cells into rabbit fetuses was without any apparent harmful effect and also resulted in permanent unresponsiveness demonstrated by donor red cell survival studies. The implication of these findings in respect of using this approach towards the correction of certain inherited diseases in man is discussed.

PMID: 6341592 [PubMed - indexed for MEDLINE]

1: J Reprod Med. 1992 Jun;37(6):515-20.

In utero stem cell therapy.

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Department of Obstetrics, Gynecology and Reproductive Sciences, University of California Medical Center, San Francisco 94143-0720.

In utero stem cell transplantation offers the potential for treating a number of genetic disorders. The combination of fetal immunotolerance and fetal marrow space makes the fetus an excellent transplant recipient. Experiments on the mouse, sheep and rhesus monkey have indicated that in utero transplantation is feasible. Human trials are currently beginning.

Publication Types:
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PMID: 1619604 [PubMed - indexed for MEDLINE]

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Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep.

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Mesenchymal stem cells are multipotent cells that can be isolated from adult bone marrow and can be induced in vitro and in vivo to differentiate into a variety of mesenchymal tissues, including bone, cartilage, tendon, fat, bone marrow stroma, and muscle. Despite their potential clinical utility for cellular and gene therapy, the fate of mesenchymal stem cells after systemic administration is mostly unknown. To address this, we transplanted a well-characterized human mesenchymal stem cell population into fetal sheep early in gestation, before and after the expected development of immunologic competence. In this xenogeneic system, human mesenchymal stem cells engrafted and persisted in multiple tissues for as long as 13 months after transplantation. Transplanted human cells underwent site-specific differentiation into chondrocytes, adipocytes, myocytes and cardiomyocytes, bone marrow stromal cells and thymic stroma. Unexpectedly, there was long-term engraftment even when cells were transplanted after the expected development of immunocompetence. Thus, mesenchymal stem cells maintain their multipotential capacity after transplantation, and seem to have unique immunologic characteristics that allow persistence in a xenogeneic environment. Our data support the possibility of the transplantability of mesenchymal stem cells and their potential utility in tissue engineering, and cellular and gene therapy applications.

PMID: 11062543 [PubMed - indexed for MEDLINE]

Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after *in utero* transplantation in sheep

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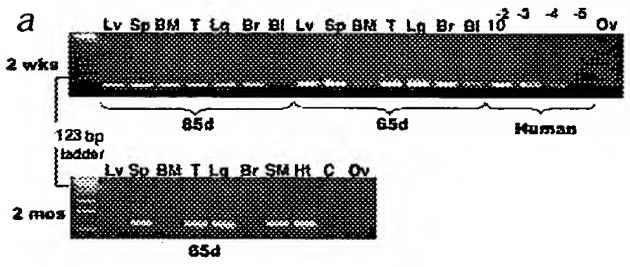
Mesenchymal stem cells are multipotent cells that can be isolated from adult bone marrow and can be induced *in vitro* and *in vivo* to differentiate into a variety of mesenchymal tissues, including bone, cartilage, tendon, fat, bone marrow stroma, and muscle[1,2]. Despite their potential clinical utility for cellular and gene therapy, the fate of mesenchymal stem cells after systemic administration is mostly unknown. To address this, we transplanted a well-characterized human mesenchymal stem cell population[3] into fetal sheep early in gestation, before and after the expected development of immunologic competence. In this xenogeneic system, human mesenchymal stem cells engrafted and persisted in multiple tissues for as long as 13 months after transplantation. Transplanted human cells underwent site-specific differentiation into chondrocytes, adipocytes, myocytes and cardiomyocytes, bone marrow stromal cells and thymic stroma. Unexpectedly, there was long-term engraftment even when cells were transplanted after the expected development of immunocompetence. Thus, mesenchymal stem cells maintain their multipotential capacity after transplantation, and seem to have unique immunologic characteristics that allow persistence in a xenogeneic environment. Our data support the possibility of the transplantability of mesenchymal stem cells and their potential utility in tissue engineering, and cellular and gene therapy applications.

Although a heterogeneous population, the human mesenchymal stem cells (MSCs) used here[3] have been well characterized in their ability to proliferate in culture with homogeneous morphology, the uniform presence of a consistent set of surface marker proteins and their differentiation into multiple mesenchymal lineages in controlled *in vitro* conditions. Analysis of colonies derived from individual cells from cultured MSCs has confirmed the presence of a subpopulation of cells with at least tri-potential differentiative capacity (bone, cartilage and adipose tissue)[3]. Assessment of the engraftment, survival and long-term fate of human MSCs after transplantation would require a surrogate animal model in which transplanted cells could be easily detected and would not be rejected. We used the fetal lamb model because it has been used as an assay system for human hematopoiesis[4]. The fetal lamb is immunologically tolerant of allogeneic skin grafts[5] or of allogeneic[6] or xenogeneic[4] hematopoietic cells before 75 days of gestation, which allows avoidance of the immunologic barriers present in post-natal models. In this model, long-term, multilineage, human hematopoietic chimerism has been established by the pre-immune transplantation of human hematopoietic stem cells. Because human and sheep DNA and proteins are widely disparate with respect to sequence homology, human-specific markers can be used for the unequivocal detection and characterization of human cells by a variety of methodologies.

To assess the influence of stage of hematopoietic development and immune response on MSC engraftment, we transplanted equivalent doses per kilogram ($1\text{-}2 \times 10^8$ MSCs/kg fetal weight, or $5\text{-}20 \times 10^6$ cells/fetus) of MSCs by intraperitoneal injection at either 65 days or 85 days of gestational age (term is 145 days). We chose these times because they represent different developmental stages in the sheep for hematopoiesis and immune response. Hematopoiesis mainly occurs in the fetal liver at 65 days of gestation, with only minimal bone marrow formation, whereas active hematopoiesis is present in the bone marrow by 85 days of gestation[7]. In addition, fetal lambs develop the capacity to reject allogeneic skin grafts[5] and demonstrate allogeneic or xenogeneic hematopoietic engraftment failure[8] after 75 days of gestation.

We assessed the early and late tissue distribution of human cells after *in utero* transplantation using PCR for human-specific β-2 microglobulin DNA sequences on DNA isolated from multiple tissues (Fig. 1). Tissues were collected at 2 weeks, 2 months, 5 months or 13 months after transplantation. We determined the tissue distribution at the time of collection of human cells transplanted at 65 and 85 days of gestation (Fig. 1*c*). All sheep transplanted at 65 days of gestation ($n = 12$) and 16 of 17 transplanted at 85 days of gestation had demonstrable human cell engraftment at the time of tissue collection. Although the pattern of human cell distribution in individual sheep differed, human-specific sequences were detectable in 28 of 29 sheep (Fig. 1*b*).

These results show that despite their large size and fibroblastic morphology, MSCs can be transplanted and are capable of engraftment in multiple tissues, even when transplanted into the fetal peritoneal cavity. This requires migration across endothelial barriers, integration into host tissue microenvironments, and survival with available growth and regulatory signals. Our finding of a variable pattern of long-term human cell engraftment after detection of human cells at 2 weeks in nearly all tissues studied supports a model of nonselective hematogenous distribution, with subsequent selective long-term survival in specific tissues. This may be a function of the ability of specific microenvironments to support the engraft-



b

	65 d	85d
2 wks	2/2	5/6
2 mos	7/7	6/6
5 mos	3/3	3/3
13 mos		2/2
	12/12	16/17

c

	2 weeks		2 months		5 months		13 months
	65 d	85 d	65 d	85 d	65 d	85 d	85 d
Lv - Liver	1/2	4/6	3/7	1/6	0/4	0/3	1/2
Sp - Spleen	1/2	5/6	1/7	2/6	1/4	0/3	0/2
BM - Bone Marrow	2/2	4/6	2/7	1/6	1/3	0/3	1/2
T - Thymus	1/2	0/6	3/7	3/6	1/4	0/3	0/2
Lg - Lung	1/2	5/6	3/7	0/6	1/3	0/3	0/2
Br - Brain	1/2	4/6	0/7	0/6	0/4	1/3	0/2
SM - Muscle	0/1	0/1	5/7	0/6	0/4	1/3	0/2
Ht - Heart	0/1	0/1	2/7	1/6	0/4	1/3	1/2
C - Cartilage	-	-	2/7	-	1/4	0/1	1/2
Bl - Blood	1/2	4/6	3/7	0/6	1/4	2/3	1/2

Fig. 1 Screening of sheep tissues (n = 29) after transplantation, by DNA PCR using probes specific for human β-2 microglobulin sequences. ***a***, Gels represent samples from two sheep transplanted at 65 d and 85 d of gestation, followed by tissue collection 2 weeks (wks) after transplantation (top), or transplanted at 65 d of gestation, followed by tissue collection 2 months (mos) after transplantation (bottom). Left lane, molecular size markers (123-base-pair ladder). Human lanes represent samples of human cells diluted in sheep cells as indicated. Tissue abbreviations as listed in panel c; Ov, ovine control DNA. ***b***, Frequency of human cell engraftment (in at least one tissue) at the time sheep were killed, assessed by PCR screening. wks, weeks; mos, months. ***c***, Distribution of human-sequence-positive tissues at the times of tissue collection for sheep in the early and late transplantation groups. –, not analyzed.

ment and differentiation of MSCs or, alternatively, the loss of engraftment from some tissues may be due to heterogeneity of the transplanted population with respect to differentiation potential or replicative capacity and longevity. A third possibility is that the xenogeneic microenvironment can support the viability and differentiation of human MSCs, but not their self-replication. We have not quantitatively assessed expansion of the cells transplanted in this experiment. As the signals that stimulate MSC proliferation and differentiation are unknown, minimal expansion of donor cells in this xenogeneic, competitive model would not be unexpected. However, the apparent low frequency of donor cells indicated by immunohistochemistry here may be misleading. In the sheep model, exponential growth of the sheep occurred after transplantation. Experiments in the sheep model of human hematopoiesis in which human cells have been quantitatively assessed in chimeric bone marrow[9,10] have shown that even when low frequencies of donor cells can be detected, there is tremendous interval expansion in the total number of donor cells since transplantation.

The apparent lack of immune response indicated by the persistence of human cells transplanted at 85 days of gestation in the sheep fetus is interesting. Potential mechanisms for tolerance include failure of immune recognition, local immune suppression or thymic clonal deletion. Human MSCs express class I human leukocyte antigen but do not express class II, which may limit immune recognition[3]. *In vitro*, MSCs added to mixed lymphocyte cultures nonspecifically ablate alloreactivity by an as-yet-unknown mechanism (K. McIntosh, personal communication). As the involvement of thymic deletional mechanisms of tolerance when foreign antigen is presented after the development of a mature T-lymphocyte repertoire is unlikely, the persistence of human cells in this model may result from a combination of minimal immunogenicity and local immune suppression.

We confirmed the presence of human cells in PCR-positive tissues by immunohistochemistry using an antibody specific for human β-2 microglobulin, a component of the class I antigen complex (Fig. 2). Negative controls, consisting of tissues from transplanted sheep that were negative by PCR and age-matched tissues from normal sheep, were uniformly negative and confirmed the human specificity of the staining (data not shown). Many human MSCs were present in pre-natal (2 months) and post-natal (5 months and 13 months) hematopoietic and lymphopoietic tissues, including the fetal liver, bone marrow, spleen and thymus (Fig. 2*a–d*). We also identified human cells by β-2 microglobulin staining in non-lymphohematopoietic sites, including adipose tissue, lung (Fig. 2*e* and *f*), articular cartilage (Fig. 3*a* and *b*), perivascular areas of the central nervous system (Fig. 3*f*), and cardiac and skeletal muscle (Fig. 4*a* and *i–k*).

We assessed differentiation of human cells in various tissues using one of four techniques: characteristic morphology by staining with antibody against human β-2 microglobulin; immunohistochemical double staining for antibody against human β-2 microglobulin and a second non-human-specific differentiation marker; a combination of *in situ* hybridization for human sequences cleaved by *Arthrobacter luteus* (*Alu*) 1 restriction endonuclease combined with non-human-specific markers of muscle differentiation; or, where available, posi-

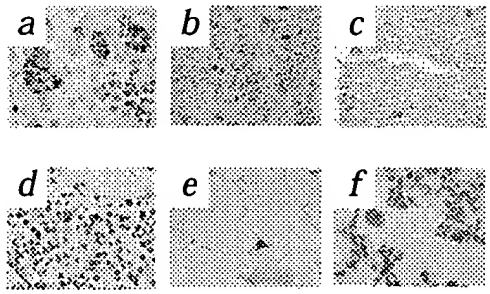


Fig. 2 Immunohistochemistry with human-specific antibody against β-2 microglobulin. ***a***, Fetal liver at 2 months after transplantation (transplanted at 65 d of gestation), showing large human cells in clusters of hematopoiesis. ***b***, Bone marrow 5 months after transplantation, showing many human cells per field. Original magnification, ×20. ***c***, Frozen section of fetal spleen 2 weeks after transplant, showing large human cells with distinct nuclei. Original magnification, ×40. ***d***, Thymus at 5 months after transplantation, showing a large human cell. ***e***, Human adipocyte at 13 months after transplantation. ***f***, Large human cell in alveolar space of fetal lung at 2 months after transplantation. Original magnification (*a* and *d–f*), ×100.

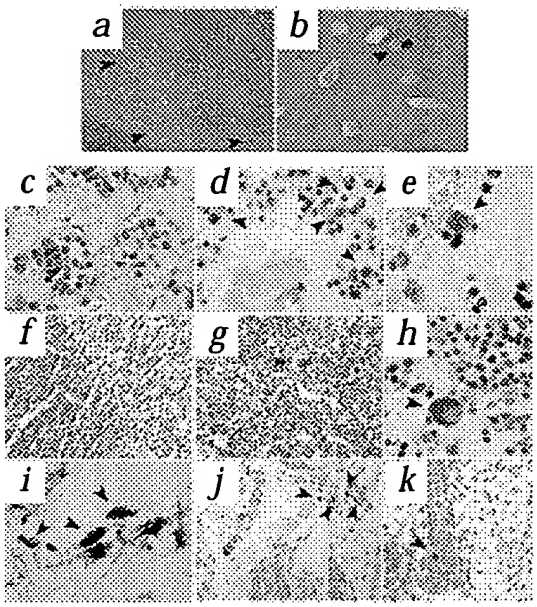


Fig. 3 Evidence for human cell differentiation. ***a*** and ***b***, Low- and high-power magnification of staining of PCR-positive articular cartilage with human-specific antibody against β-2 microglobulin. ***a***, Human cells in three separate lacunae of the cartilage (arrowheads) . ***b***, Higher-power magnification of human lacunar cell (arrowhead). ***c–e***, Staining of bone marrow 5 months after transplantation with human-specific CD23. ***c***, Negative control marrow from age-matched normal sheep. ***d***, CD23 staining of PCR positive bone marrow, showing many large human cells. ***e***, High-power magnification of CD23-positive bone marrow cells (arrowhead). ***f–h***, Staining of thymus 5 months after transplantation with human-specific CD74. ***f***, Negative control thymus from age matched normal sheep. ***g***, Low-power magnification of CD74-positive cell (arrowhead). ***h***, High-power magnification of a human thymic stromal cell (arrowhead). Original magnification, 200×. ***i***, Double staining of PCR-positive brain tissue with anti β-2 microglobulin (dark brown granules, arrowheads) and anti glial fibrillary acid protein (GFAP-purple). ***j***, Staining with antibody against β-2 microglobulin at 2 months after tail wounding. Multiple human cells are seen at the wound in the dermis (arrowheads) and in dermil appendages, ***k***.

tive staining with human-specific differentiation markers proven not to cross-react with sheep cells. We confirmed site-specific differentiation for many cell types. We identified chondrocyte differentiation by finding human β-2 microglobulin-positive cells in the lacunae of articular cartilage (Fig. 3*a* and *b*). The immunohistochemical identification of human cells within the lacunae of cartilage specimens that were PCR-positive for human β-2 microglobulin sequences represented definite evidence of human chondrocyte differentiation. We identified human adipocytes in PCR-positive specimens by staining with antibody against human β-2 microglobulin and characteristic morphology (Fig. 2*e*). We assessed differentiation of human MSCs in bone marrow by immunohistochemistry using a human-specific antibody against CD23. CD23 is the low-affinity immunoglobulin E receptor and is expressed on a variety of cell types, including B-cell progenitors and bone marrow stromal cells[11]. At 2, 5 and 13 months after *in utero* transplantation, many human cells were present in the marrow and expressed CD23 (Fig. 3*d* and *e*). These human CD23-positive cells seemed to be large cells clustered in areas with ovine hematopoietic elements, consistent with bone marrow stroma. Staining of CD23-positive bone marrow with antibody against human CD45 was consistently negative, confirming that the human cells in the bone marrow were non-hematopoietic. We assessed differentiation of human MSCs in the thymus using immunohistochemistry with a human-specific antibody against CD74. At 2 and 5 months after *in utero* transplantation, multiple human cells detected in the thymus strongly expressed CD74 (Fig. 3*g* and *h*), a major-histocompatibility-complex-associated invariant chain expressed on thymic stromal cells[12]. These cells were large and were similar in morphologic appearance to epithelial cells, supporting our interpretation of the cells as thymic epithelial cells. The precursor of thymic dendritic cells is thought to be the hematopoietic stem cell, whereas the origin of the thymic epithelial cell is controversial. Our data supports but does not prove a mesenchymal origin for the thymic epithelial cell as a stromal-supporting cell in the thymus. Although human cells in the central nervous system were identified by PCR, these cells were located in the perivascular spaces in the gyral sulci rather than in the brain parenchyma and, on double staining, did not co-localize with cells expressing glial fibrillary acid protein, a marker for glial cell differentiation (Fig. 3*i*). Finally, we documented cardiomyocyte and skeletal myocyte differentiation by a combination of approaches. In cardiac muscle, β-2 microglobulin staining (Fig. 4*a*) or *in situ* hybridization

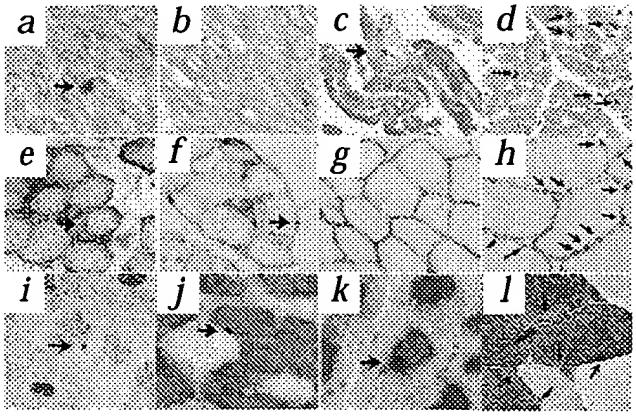


Fig. 4 Evidence for cardiac and skeletal myocyte differentiation. ***a–d***, Cardiac muscle assessed at 5 months. ***a***, Human cell (arrow) in the heart identified using antibody against β-2 microglobulin (dark brown) and counterstaining (pink) with antibody against SERCA-2. Original magnification, ×100. ***b***, Age-matched sheep heart; negative control for staining with antibody against β-2 microglobulin and counterstaining with SERCA-2. ***c*** and ***d***, *In situ* hybridization for human *ALU* sequences and counterstaining with SERCA-2. ***c***, Human nucleus (arrow) within sheep cardiomyocyte. ***d***, Human heart (positive control). ***e–h***, Skeletal muscle analyzed by *in situ* hybridization for human ALU sequences and counterstained with dystrophin. ***e*** and ***f***, Sheep skeletal muscle in cross-section with human nuclei (arrows) on the periphery of the cell. ***g***, Age-matched sheep muscle (negative control). ***h***, Human skeletal muscle (positive control). The arrows show human nuclei identified by *in situ* hybridization for human *ALU* sequences. ***i–l***, Skeletal muscle stained with human-specific antibody against β-2 microglobulin and various counterstains. ***i***, Counterstaining with SERCA-2, showing a human nucleus (arrow) on the periphery of a myocyte. ***j***, Counterstaining with antibody against slow myosin, showing a human nucleus (arrow) on the periphery of a myocyte. ***k***, Counterstaining with antibody against fast myosin, showing a human nucleus (arrow) on the periphery of a myocyte. ***l***, Human skeletal muscle (positive control), counterstained with SERCA-2.

for human *ALU* sequences (Fig. 4*c*) were combined with double staining with antibody against smooth endoplasmic reticulum ATPase-2 (SERCA-2), a cytoplasmic protein specific for smooth or skeletal muscle[13]. We confirmed skeletal muscle differentiation by staining with antibody against dystrophin[14] combined with *in situ* hybridization for human ALU sequences (Fig. 4*e* and *f*), or by double staining for β-2 microglobulin and SERCA-2 (Fig. 4*i*), fast myosin (Fig. 4*j*) or slow myosin (Fig. 4*k*).

We also localized human cells at the site of wounds (tail clipping) inflicted at the time of MSC transplantation. These cells were in the dermis and dermal appendages and had fibroblastic features, indicating possible participation in wound healing (Fig. 3*j* and *k*).

Many reports have documented donor-derived stromal elements after bone marrow transplantation[15,16], indirectly supporting the possibility of the presence of a 'non-hematopoietic' stem cell in whole bone marrow that gives rise to stromal supporting elements. Similarly, a report documenting donor derived skeletal muscle in a muscle injury model after bone marrow transplantation[17] supports the possibility of a bone-marrow-resident stem cell with mesenchymal differentiative capacity. Evidence supporting the presence of osteoprogenitors in bone marrow has been reported in clinical[18] and experimental[19] studies. All of these studies used whole bone marrow as a donor source rather than more-defined 'stem cell' populations. Muscle reconstitution has been shown in irradiated mice with X-linked muscular dystrophy (*mdx*) after transplantation of highly enriched hematopoietic stem cells[20]. Those findings[20] indicate that the MSC may be an intermediate population and that the hematopoietic stem cell is more pluripotent than previously realized. More relevant here are studies that monitored the fate of MSCs or MSC-like populations after intravenous or intraperitoneal transplantation. In two studies in mice, cultured mouse adherent cell populations systemically transplanted persisted after transplantation[21,22]. Donor cells were detected in bone marrow, spleen, bone, cartilage and lung up to 5 months later by PCR or fluorescence *in situ* hybridization assays for the Y chromosome. Although these studies support the possibility of the presence of a bone-marrow-resident MSC, our study has directly documented multipotential differentiation of a relatively well-characterized MSC population *in vivo* after transplantation. Our finding of long-term persistence of MSCs with multipotential, site-specific differentiation supports the potential of these cells in transplantation, gene therapy and tissue engineering applications. However, clinical use will require the delivery of adequate numbers of MSCs to specific sites for therapeutic effect. This will require further insight into normal and disease-induced regulation of MSC proliferation and differentiation, a better understanding of the transplant immunology of MSCs and the development of strategies for diffuse or site-specific delivery for therapeutic applications.

Methods

Mesenchymal stem cell isolation and preparation. Fresh bone marrow was obtained by iliac crest aspiration from normal human donors after informed consent was given. MSCs were isolated as described[3]. Bone marrow aspirate (10 ml) was added to control medium (20 ml Dulbecco's modified essential media; Life Technologies) containing 10% FBS (Hyclone, Logan, Utah) from selected lots, and was centrifuged to pellet the cells and remove the fat. The cell pellet was resuspended in control medium and fractionated on a density gradient generated by centrifugation of a 70% percoll solution (Sigma) at 13,000*g* for 20 min. The MSC-enriched, low-density fraction was collected, rinsed with control medium and plated at a density of 1×10^7 nucleated cells per 60-mm^2 dish. The MSCs were cultured in control medium at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching near confluence, the cells were detached for 5 minutes at 37 °C with 0.25% trypsin containing 1 mM EDTA (Life Technologies). The cells were washed with control medium and were resuspended at a density of 5×10^5 MSCs/ml in control medium containing 5% DMSO (Sigma). The cells were then stored in liquid nitrogen.

Animals and transplant procedure. Pregnant Western Cross sheep (Thomas Morris, Reisterstown, Maryland) carrying twin pregnancies of confirmed gestational age were housed in the large animal facility at the Children's Hospital of Philadelphia, approved by the Assocoation for Assessment and Accreditation of Laboratory Animal Care (ALAAC). After induction of anesthesia and exposure of the fetus by maternal laparotomy and hysterotomy, MSCs were injected (1×10^6–2×10^6 human MSCs/kg estimated fetal weigh), with direct visualization, into the fetal peritoneal cavity.

Tissue processing. Fetal sheep were killed at 2 weeks or 2, 5 or 13 months after injection, and liver, spleen, lung, bone marrow, thymus, brain, heart, skeletal muscle, cartilage and blood were collected and analyzed for the presence of human cells by DNA isolation and immunohistochemistry. Tissues positive by PCR were analyzed by immunohistochemistry. Tissues negative by PCR and tissues from normal age-matched sheep were used as negative controls. In a subset of 65-day gestation recipients, the fetal tails were cut off at the time of MSC injection, and the tail wounds were obtained at 1 week or 2 months after wounding. Fetal tissues were fixed overnight at 4 °C in 10% neutral buffered formalin (Fisher Scientific). Bone marrow samples were then decalcified in Cal-EX (Fisher) for 12 h to 2 weeks (depending on size), followed by a 30-minute wash in 3% H_2O_2, and a 1-hour wash with distilled water. Samples were then embedded in paraffin as described[23]. In addition, samples from each tissue were 'snap-frozen' in liquid nitrogen and stored at -80 °C for subsequent total cellular DNA extraction. Skeletal muscles were frozen in isopentane-chilled in liquid nitrogen for subsequent frozen section analysis.

DNA Isolation. Total cellular DNA was isolated using DNAzol (Molecular Resource Center, Cincinnati, Ohio). Approximately 100 mg tissue was homogenized in 1 ml DNAzol. The DNA was precipitated with 0.5 ml 100% ethanol. The DNA precipitate was pelleted by centrifugation and then washed twice with 95% ethanol. The DNA pellet was then dissolved in sterile water at a concentration of 15 μg/ml.

PCR analysis. Total cellular DNA was analyzed by PCR for human-specific β-2 microglobulin using a modification of published methods[24]. Specific primers for human β-2 microglobulin were selected based on the published human sequence (upstream primer, 5′-GTGTCTGGGTTTCATCAATC-3′; downstream primer, 5′-GGCAGGCATACTCATCTTTT-3′) and were shown to specifically amplify human, not ovine, DNA. Amplification conditions were: 95 °C for 9 min, followed by 45 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 15 s, followed by an extension at 72 °C for 5 min. All samples were also amplified to detect the β-actin gene (upstream primer, 5′-CGGGACCTGACTGACTAC-3′; downstream primer, 5′-GAAGGAAGGCTGGAAGAG-3′) as a control for the presence of amplifiable DNA. For these controls, the reaction conditions were the same except 35 cycles of amplification were used. The sensitivity of the assay was assessed by human/sheep cell dilution studies with a sensitivity of detection of 1 human cell in 10,000 cells.

Immunohistochemistry. Immunohistochemical staining was also done for human CD74, human CD23, SERCA-2, dystrophin, myosin heavy chain (fast and slow) and glial fibrillary acid protein. For all tissues except skeletal muscle, paraffin sections 4–5 μm in thickness were collected on Superfrost Plus slides (Fisher) from each of the

paraffin-embedded tissues. Slides were incubated for 16 h at 55 °C and then deparaffinated by immersion in xylene followed by rehydration over 10 min through a graded alcohol series to deionized water. To enhance antigen retrieval, the slides were immersed in 1% Antigen Unmasking Solution (Vector Laboratories, Burlingame, California) and microwaved for 3 min. Samples were blocked for 30 min at room temperature with non-immune serum from the species in which the primary antibody was raised (1:10 dilution), followed by a 16-hour incubation with the specific primary antibody. The primary antibodies and dilutions used were: human β-2 microglobulin, 1:50–1:400 (PharMingen, San Diego, California); human CD74, 1:20 (Novocastra Laboratories, Newcastle upon Tyne, UK); and human CD23, 1:20 (Vector Laboratories). The slides were then washed with PBS followed by a second blocking step of 30 min at room temperature in methanol containing 0.6% hydrogen peroxide. Slides were then rinsed with deionized water and then PBS, followed by incubation for 30 min at room temperature with biotinylated secondary antibody (1:200 dilution; Vectastain ABC kit AK-5002; Vector Laboratories). The slides were washed with PBS, and avidin–biotin complex was added for 30 min at room temperature. The slides were then rinsed well in PBS and developed with chromagen 3,3′-diaminobenzidine. For sections stained for human β-2 microglobulin, CD74 and CD23, the slides were then lightly counterstained with hematoxylin. For cardiac muscle and brain, the human β-2 microglobulin was first developed using nickel chloride as the chromagen and then was subjected to a secondary immunohistochemical staining for SERCA-2 (1:50 dilution) or glial fibrillary acid protein (1:100 dilution), respectively (both from Vector Laboratories), as described[25]. Secondary staining was developed using Vector VIP substrate kit (Vector Laboratories). No counterstaining was used on these double-stained slides.

For skeletal muscle frozen sections, sections 5 μm in thickness were collected on glass slides and stored at –20 °C. Slides were air-dried and incubated overnight at 4 °C or at 37 °C for 3 h with primary antibody: human dystrophin (clone DYS-2; 1:10 dilution), human myosin heavy chain, slow type (clone WB-MHCs; 1:100 dilution) or human myosin heavy chain, fast type (clone WB-MHCf; 1:100 dilution, all from Novocastra Laboratories). Slides were then incubated with secondary antibody and avidin–biotin complex as described above, except that the Vector alkaline phosphatase mouse IgG kit (PK-6102; Vector Laboratories) was used and washes were done in 0.1 M Tris-buffered saline, pH 7.5. The slides were developed using the Vector alkaline phosphatase substrate kit (SK-5200; Vector Laboratories). For double staining with β-2 microglobulin, slides were transferred to distilled water, then fixed for 30 min at room temperature in 1% neutral buffered formalin (Fisher Scientific) in PBS. They were blocked for 30 min at room temperature with 10% horse serum, followed by incubation with human β-2 microglobulin antibody as described above.

In situ hybridization. For frozen skeletal muscle sections, slides were first stained with dystrophin or myosin antibodies and then developed as described above before the *in situ* hybridization. Slides were incubated for 5 min with 100 μg/ml proteinase K, then were post-fixed in 1% formalin in PBS. A fluorescein-conjugated human ALU probe (Innogenex, San Ramon, California) was applied and hybridization was done at 80 °C for 5 min, followed by 37 °C overnight. Slides were developed using the Innogenex *in situ* hybridization kit for fluorescein-labeled probes (SH-2009-06; Innogenex) according to the manufacturer's directions. After the horseradish peroxidase step, the Vector DAB substrate kit (SK-4100; Vector Laboratories) was used to develop the stain. For cardiac sections, *in situ* hybridization was done before staining with SERCA-2. Slides were deparaffinated, hydrated and 'microwave retrieved' as described above, then incubated for 20 min at 37 °C with 20 μg/ml proteinase K. The remainder of the staining was as for the frozen muscle sections except a 2× concentration of the human ALU probe was used (Innogenex).

RECEIVED 28 MARCH; ACCEPTED 22 SEPTEMBER 2000

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Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta

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In principle, transplantation of mesenchymal progenitor cells would attenuate or possibly correct genetic disorders of bone, cartilage and muscle, but clinical support for this concept is lacking. Here we describe the initial results of allogeneic bone marrow transplantation in three children with osteogenesis imperfecta, a genetic disorder in which osteoblasts produce defective type I collagen, leading to osteopenia, multiple fractures, severe bony deformities and considerably shortened stature. Three months after osteoblast engraftment (1.5–2.0% donor cells), representative specimens of trabecular bone showed histologic changes indicative of new dense bone formation. All patients had increases in total body bone mineral content ranging from 21 to 29 grams (median, 28), compared with predicted values of 0 to 4 grams (median, 0) for healthy children with similar changes in weight. These improvements were associated with increases in growth velocity and reduced frequencies of bone fracture. Thus, allogeneic bone marrow transplantation can lead to engraftment of functional mesenchymal progenitor cells, indicating the feasibility of this strategy in the treatment of osteogenesis imperfecta and perhaps other mesenchymal stem cell disorders as well.

Bone marrow contains not only precursors for the hemopoietic system, but also cells that can give rise to mesenchymal lineages, including bone, cartilage and muscle[1–6]. In preclinical models, transplanted marrow-derived mesenchymal cells migrated to and became incorporated into bone and muscle of the recipient animals, indicating that these cells have a 'homing' capacity[8–11]. In principle, therefore, bone marrow transplantation could be used to correct a far wider range of inherited and acquired disorders than now occurs. Support for this has come almost exclusively from murine models, leaving several principal questions unanswered. It is unknown, for example, whether mesenchymal cells from human marrow are capable of engrafting in allogeneic hosts and whether such cells can differentiate and function normally *in vivo*. To begin to address these questions, we undertook bone marrow transplantation in three patients with osteogenesis imperfecta.

Osteogenesis imperfecta (OI) is a genetic disorder of mesenchymal cells in which generalized osteopenia leads to bony deformities, excessive fragility with fracturing, and short stature. The underlying defect is a mutation in one of the two genes encoding type I collagen, the primary structural protein of bone[12]. There is no cure for OI, nor is there any proven therapy for alleviating its symptoms[12–14]; although pamidronate, one of the bisphosphonate compounds, may have some therapeutic potential[15]. We chose this disorder to validate the principle of mesenchymal progenitor cell transplantation because engraftment of mesenchymal cells in a murine model of OI produced a small but appreciable improvement in the disease phenotype[16]. Here we demonstrate that marrow-derived mesenchymal cells can indeed engraft in humans and generate donor-derived osteoblasts that function sufficiently well for 6 months, to attenuate the biochemical, structural and clinical abnormalities associated with OI.

Engraftment of hemopoietic and mesenchymal cells

Three children with severe deforming OI (Table) were intravenously infused with unmanipulated bone marrow from HLA-identical or single-antigen-mismatched siblings after they had received ablative conditioning therapy. All three showed engraftment with hemopoietic donor cells. Patient 1 had a mixed hemopoietic chimerism (21% donor cells) that was stable for more than 6 months. In patients 2 and 3, more than 99% of the hemopoietic cells analyzed were of donor origin. Osteoblasts were cultured from fresh bone biopsy specimens. The individual adherent cells in the cultures had typical osteoblast morphology, expressed alkaline phosphatase and produced stainable matrix. Flow cytometric analysis of these cells indicated a lack of conta-

Table Characteristics of the patients and the transplantation protocol

Patient	Age[a]/sex	Weight	Length	Head circum. (percentile)	Genetic mutation	Conditioning regimen[d]	Total nucleated cell dose[e]
1	13/F	5.04 kg (2.5[b])	54.5 cm (1.5[c])	45.5 cm (45th)	Gua→Ade in exon 38 of *COL1A1*; Gly→Ser at residue 688 of proα1(I)	Busulfan (1 mg/kg × 16) Ara-C (2 g/m² × 6) Cyclo (45 mg/kg × 2)	5.7×10^8 cells/kg
2	13/M	5.04 kg (2[b])	56.0 cm (1.5[c])	44.5 cm (10th)	18 base-pair duplication in *COL1A2*; duplication of residues 899–904 of proα2(I)	Busulfan (40 mg/m² × 8) Cyclo (60 mg/kg × 2) TBI (180 cGy × 5)	6.2×10^8 cells/kg
3	32/M	9.00 kg (8.5[b])	70.0 cm (7.5[c])	50.4 cm (50th)	Ade→Cyt, 4 bases downstream of exon 41 in *COL1A1*; splice mutant	Busulfan (1 mg/kg × 16) Cyclo (50 mg/kg × 4)	7.5×10^8 cells/kg

[a]In months. [b]Median age (in months) for weight in the general pediatric population[21]. [c]Median age (in months) for length in the general pediatric population[21]. [d]Ara-C, cytarabine; Cyclo, cyclophosphamide; TBI, total body irradiation (added because sibling donor was a HLA DRβ1 mismatch). [e]Total number of nucleated cells in unmanipulated donor bone marrow per kg of recipient weight.

minating lymphohemopoietic cells (Fig. 1). Fluorescence *in situ* hybridization to detect the Y chromosome in osteoblasts collected from patient 1 on day 101 after transplantation showed that 1.5% of the cells were of donor origin. DNA polymorphism analysis of osteoblasts from patient 2, collected on day 80 after transplantation, demonstrated 2.0% donor cells (Fig. 2). Osteoblasts could not be grown from patient 3, precluding evaluation of engraftment with donor-derived mesenchymal cells.

Changes in bone histology and mineral content

Engraftment was associated with improvements in bone histology (Fig. 3). A specimen of trabecular bone taken before transplant from the iliac wing of patient 1 contained numerous, disorganized osteocytes, enlarged lacunae and relatively few osteoblasts (Fig. 3*a*). The bone had the characteristic appearance of high bone turnover, including woven bone, which is characteristic of OI (refs. 17–19)(Fig. 3*e*). Fluorescence microscopy of the same specimen showed a distorted pattern of tetracycline labeling (Fig. 3*c*), consistent with the disorganized formation of new bone and poor mineralization. In contrast, similar specimens taken on day 216 after transplantation, near the site used previously, showed a reduced number of osteocytes, linearly organized osteoblasts and evidence of lamellar bone formation (Fig. 3*b* and *f*). Because of fragmentation of the specimens, full histomorphometric analysis could not be done; however, using a magnification of ×100, we counted the number of osteoblasts per high-power field in biopsy specimens taken both before and after transplantation (five fields each). There were 4.6 ± 1.8 (s.e.m.) osteoblasts per high-power field in the sample taken before transplantation, compared with 16.0 ± 3.0 in the sample taken after transplantation ($P = 0.005$, t-test). Fluorescence microscopy showed linear, single and double tetracycline labeling, indicative of improved bone formation and mineralization. Similar histologic changes were apparent in biopsy specimens from patients 2 and 3, taken from the iliac wing opposite the initial specimen (not shown).

There was also an increase in the total body bone mineral content, as determined by measurements with dual energy X-ray absorptiometry. The three patients accumulated 21–29 g of bone mineral (median, 28 g) during the first 100 days after transplantation in the absence of substantial weight gains and with only small changes in body length (Fig. 4*a*). In contrast, normal children would not be predicted to show changes in bone mineral content in only 100 days without substantial changes in body mass[20].

The most salient result was a 77% increase in total body bone mineral content (21.6 g, from 28.0 to 49.6 g) in patient 1, who grew 2.5 cm and did not gain weight during the first 100 days after transplantation. Patient 2 had a less salient but still considerable 45% gain (29.8 g, from 67.0 to 96.8 g). This 13-month-old boy grew 2.0 cm and did not gain weight after transplantation. Although patient 3 lacked interpretable absolute measurements of total body bone mineral content, because of the presence of three intramedullary rods, he did gain 28.6 g of bone mineral with minimal weight gain and grew 0.5 cm, in agreement with findings in patients 1 and 2.

Clinical correlates of improved osteogenesis

During the 13 months immediately preceding bone marrow transplantation, patient 1 had 37 documented fractures, and patient 2 had 20. During the first 6 months after transplantation, only three fractures were identified in patient 1 by clinical assessment and radiographic skeletal survey, and only two were found in patient 2. Patient 3, who had rods in both femurs and in the right humerus, had three fractures during the 6 months just before transplantation, but none during the next 6 months.

From 6 to 13 months of age, patients 1 and 2 achieved only 50% and 40%, respectively, of the normal median growth velocity for age- and sex-matched children[21]. After transplantation, patient 1 grew 8.0 cm in 7 months, and patient 2 grew 6.5 cm in 6 months, approximately 100% of the normal median growth velocity. About two-thirds of this growth occurred after 100 days after transplantation. These results contrast with the usual slowing of growth in children of this age group who have type III OI (ref. 22). Patient 3, who was 19 months older than the other two patients, failed to grow during the 6 months preceding transplantation, but during the next 6 months, he grew 1.5 cm, or 38% of the normal median velocity (Fig. 4*b*).

Toxicity

Neither patient 1 nor patient 3 had clinically significant toxicity over the transplantation course. The course for patient 2 was more complex, including sepsis, pulmonary insufficiency and the development of a bifrontal hygroma. Although the first two complications are recognized risks of bone marrow transplantation, an association with bifrontal hygroma has not been described. Whether transplantation produces unique neurologic complications in children with OI remains to be determined. All of the complications in patient 2 have resolved, and the child is doing well.

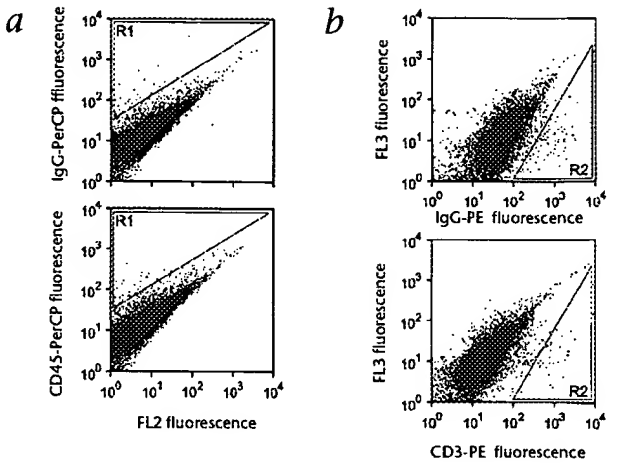

Fig. 1 Flow cytometric analysis of cultured osteoblasts to exclude the presence of contaminating lymphohemopoietic cells. The scatter plots were constructed from data collected on 50,000 cells. ***a***, Screening for leukocytes with anti CD45-perCP (peridinin chlorophyll protein). The R1 gate of the isotype control (upper) contains 0.06% of the cells; the gate of the experimental analysis (lower) contains 0.10%. ***b***, Screening for T lymphocytes with anti-CD3-PE (phycoerythrin). The R2 gate for the isotype control (upper) contains 0.18% of the cells; the gate of the experimental analysis (lower) contains 0.07%.

Discussion

Bone marrow transplantation has not been considered as a means to correct disorders of mesenchymal cells. Its successful use to treat children with osteopetrosis[23] can be explained by the hemopoietic (rather than mesenchymal) stem cell origin of the osteoclast, the defective cell in this genetic bone disorder[24]. Our study demonstrates that mesenchymal progenitors in transplanted marrow can migrate to bone in children with osteogenesis imperfecta, and then give rise to osteoblasts whose presence correlates with an improvement in bone structure and function. Engraftment of mesenchymal cells in OI patients might be possible because the normal osteoblasts derived from the allograft compete successfully with the recipient's cells that express the genetic defect. Although the percent increases in total body bone mineral content and actual linear growth velocities differed among the three patients, the incremental gains in mineral content were similar, in agreement with the similar doses of nucleated marrow cells received by these patients (Table). This indicates that increased mineralization may be related to mesenchymal cell dose. Finally, the accelerated growth velocity shown by each child during the first 6 months after transplantation contrasts with the typical findings in OI patients[22] and with the usual clinical course in patients undergoing marrow transplantation for disorders other than OI, in whom there is maintenance or slowing of growth[25–28].

Selection of OI as a prototypic bone disorder was prompted mainly by observations that mesenchymal progenitors can migrate to and become incorporated into bone in murine models[11,16]. Moreover, studies of the parents of probands with lethal OI indicated that some parents were mosaic for the same mutation in type I procollagen that produced severe OI in the offspring[29]. The mosaic parents were asymptomatic even though the ratio of mutated-to-normal alleles in some tissues, including skin fibroblasts, approached the value of 1:1 seen in the tissues of their affected offspring. This finding indicates that the severity of the disease is essentially dependent on the relative balance between the rates of synthesis of mutated and normal proα polypeptide chains. Indeed, different lines of transgenic mice that expressed various levels of the same mutated COL1A1 gene showed a range of OI manifestations[30]. Therefore, even low levels of mesenchymal progenitor cell engraftment may be sufficient to produce a shift in the balance between the synthesis of mutated and normal proα chains, thereby converting a severe OI phenotype to a less-severe one.

This prediction helps to explain how the presence of only 1.5–2.0% donor mesenchymal cells in our patients could lead to improvements in total body bone mineral content, body growth, fracture incidence and bone histology. The principle that low-level correction of a genetic defect can produce clinical benefit is well-known in such diseases as chronic granulomatous disease[31] and osteopetrosis[23]. Another consideration is that the presence of a small fraction of normal osteoblasts could alter the osteogenic microenvironment, as has been suggested in a study of osteopetrosis[32]. This explanation might also account for the greater than expected increase in osteoblasts at 6 months after transplantation.

An alternative is that engraftment may have been short-lived;

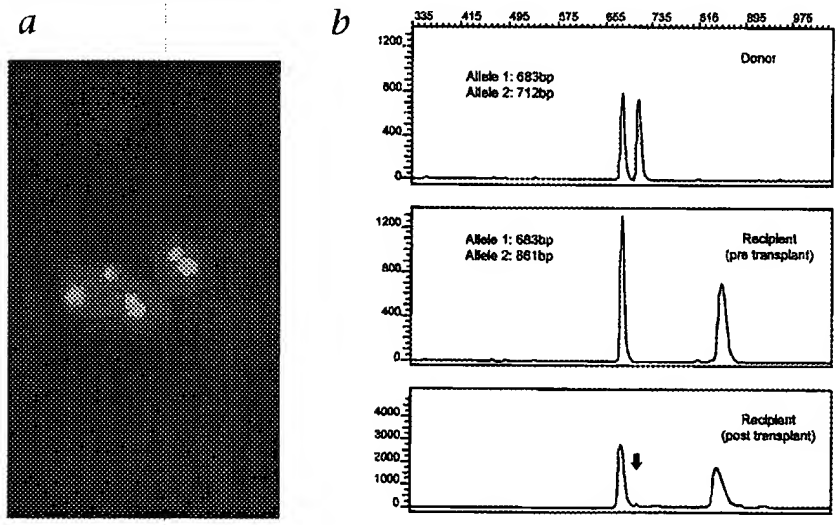

Fig. 2 ***a***, Fluorescence *in situ* hybridization analysis of interphase nuclei from the cultured osteoblasts of patient 1 on day 101 after transplantation. Both X (red) and Y (green) chromosomes are present in one of the cells from this female patient. Of the cells studied, 1.5% were of donor (male) origin; of the 500 female control cells counted, all demonstrated an XX pattern. ***b***, Electropherograms based on an analysis of DNA polymorphisms of the donor (top) and patient 2 (middle) before transplantation, and of osteoblasts from the patient on day 80 after transplantation (bottom). The peak indicated by the arrow represents about 2% donor cells.

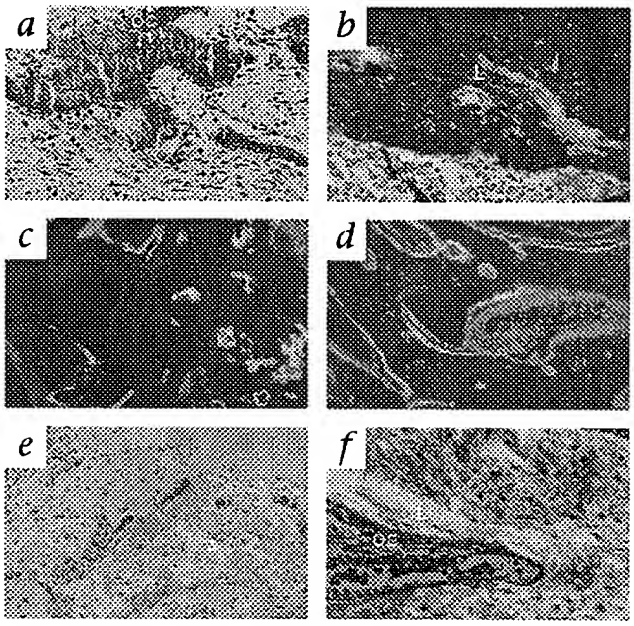

Fig. 3 *a*, Biopsy specimen of trabecular bone before transplantation, stained with Goldners-Masson trichrome. The calcified tissue appears blue-green, and the uncalcified tissue is red-brown. Numerous, randomly arranged osteocytes (OC) are present in large lacunae. There are also peritrabecular marrow fibrosis, a paucity of osteoblasts relative to the specimens after transplantation and an incompletely calcified area of bone matrix. *b*, A specimen after transplantation stained with Goldners-Masson trichrome, taken near the site shown in Fig. 2*a*. There are fewer osteocytes, and there is a small section of lamellar bone (L), indicating normalization of the remodeling process. Original magnification, ×88. *c*, Fluorescence photomicrograph of the tetracycline-labeled trabecular bone specimen (same section as in Fig. 2*a*). The labeling is poorly defined, indicating disorganized formation of new bone and abnormal mineralization. *d*, A contrasting specimen after transplantation with definitive, crisp, single and double tetracycline labeling, indicative of considerably improved new bone formation and mineralization. Original magnification, ×56. *e*, Trabecular bone specimen before transplantation, stained with toluidine blue and photographed under polarized light to emphasize the woven (w) texture of the bone, a characteristic feature of patients with osteogenesis imperfecta[17–19]. *f*, Bone specimen after transplantation stained with toluidine blue and photographed under polarized light, demonstrating lamellar bone (L) formation, and linearly arranged osteoblasts (OB) in areas of active bone formation along the calcified trabecular surface. Original magnification, ×88.

hence, the percentage of donor osteoblasts early after engraftment may have been greater than during the cell sampling interval (at 80–101 days after transplantation). The increased amounts of normal collagen fibers deposited by these cells might well have provided a matrix for the deposition of mineral, resulting in stronger bone that persisted longer than most donor osteoblasts. This could also explain our observation that each child's total body bone mineral content increased with minimal changes in height and weight. Although each child did grow between the time of transplantation and the aborptiometry scan, we attribute the increase of bone mineral content to enhanced mineralization of existing bone, possibly because of an improved ratio of normal to mutated collagen.

A third explanation for the therapeutic effects observed after low-level mesenchymal cell engraftment is that the changes were independent of the donor osteoblasts, and were induced by the allogeneic transplantation procedure itself. However, this seems unlikely, as similar effects have not been observed in animal models[11,16], and total-body irradiation and the cytotoxic drugs used for allogeneic transplantation generally have inhibitory (not stimulatory) effects on growth and development of children[24–28].

The ultimate value of bone marrow transplantation for OI will depend on the ability to infuse adequate quantities of mesenchymal progenitors and to devise clinical protocols that are both safe and easy to follow. It will also be important to extend follow-up studies to learn whether early improvements in bone structure and function reflect the activity of self-renewing stem cells or merely that of progenitors with limited proliferative capacity. Finally, the therapeutic activity of donor mesenchymal progenitor cells in patients with OI indicates that bone marrow transplantation may also be feasible in other disorders originating in mesenchymal progenitors, such as hypophosphatasia[33] or possibly even muscular diseases[8].

Methods

Patients and transplantation procedure. All three children with OI were enrolled (with informed parental consent) in a clinical trial that had been approved by the Institutional Review Board of St. Jude Children's Research Hospital. Each patient had a mutation of either the *COL1A1* or *COL1A2* gene that is associated with severe deforming (type III) OI and had physical features indicative of poor bone growth and development (Table).

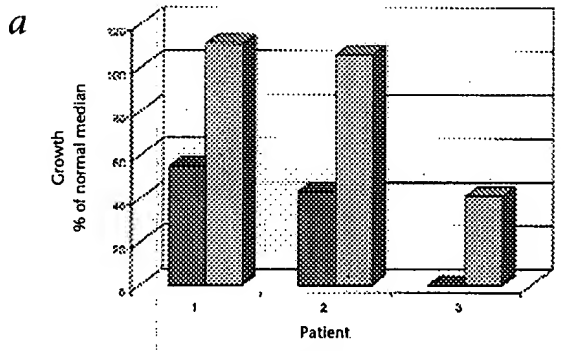

Fig. 4 *a*, Growth rates of the patients during the 6 months immediately before (▩) and after (▨) transplantation. The values are percentages of the median growth of unaffected children of the same age and sex. *b*, Increase of total body bone mineral content (TBBMC) at approximately 100 days after transplantation (▨). Dual-energy X-ray absorptiometry scans, obtained just before transplantation, served as the baseline. The predicted increase of TBBMC (ref. 20; ▩), based on an increase (if any) in the child's weight, is shown for comparison.

The bone marrow transplantation conditioning regimens received by these patients are outlined in the Table. Moderate-dose total-body irradiation was added to the regimen for patient 2 because of a mismatch at the HLA DRβ1 allele with his sibling donor. Mesna was always administered with cyclophosphamide, and phenytoin with busulfan. Unmanipulated bone marrow freshly harvested from a sibling donor was intravenously infused into each patient. Chemoprophylaxis against graft-versus-host disease consisted of intravenous cyclosporine (2.5 mg/kg every 12 hours), begun 2 days before transplantation.

Growth evaluation. Each patient was measured from crown to heel by the same investigator (P.L.G.) before and at 6 months after transplantation. Growth velocity was determined as the difference between measurements at these two intervals, which were chosen to avoid any confounding effects of growth spurts and plateaus. Angulations of the bony deformities, which can alter direct measurements of body length, were unchanged over the 6-month observation period, as indicated by a radiographic skeletal survey.

Bone histologic studies. Patients received 3-day courses of tetracycline at approximately 3 weeks and 1 week before biopsy. A 5.0-mm core of iliac bone was taken before and 6 months after transplantation with a trephine inserted through a 1.5 cm incision, from patients sedated by general anesthesia. Histologic changes were determined on sections 5 μm in thickness of polymethyl methacrylate-embedded samples, using a Zeiss microscope.

Mesenchymal cell cultures. Osteoblasts from bone biopsies were prepared and maintained in culture as specifically described for this cell type by Robey and Termine[34]. Bone fragments were dissected from soft tissue, progressively 'minced' to a fine granular consistency, digested with collagenase and placed into culture. Flow cytometric analysis indicated a lack of lymphohemopoietic cells in the osteoblast preparations (Fig. 1).

Chimerism studies. For sex-mismatched donor-recipient pairs, peripheral blood or cultured mesenchymal cells (passage 1) were analyzed by fluorescence *in situ* hydridization to determine the sex chromosome ratio, according to the manufacturer's suggestions (VYSIS, Downers Grove, Illinois). For sex-matched pairs, chimerism was determined by analysis of DNA polymorphisms between donor and recipient (PCR amplification of a variable number of tandem-repeat sequence) by collaborators at the Molecular Diagnostics Laboratory, University of Minnesota.

Dual-energy X-ray absorptiometry. These measurements of total body bone mineral content were done on a whole-body scanner with a pediatric platform (Hologic QDR 2000 Densitometer; Hologic, Inc., Waltham, Massachustetts), as described[20,35,36].

Acknowledgments

We acknowledge J. Marini for discussions throughout this study; and S. Nooner, W. Cabral, B. Hopkins and M. Kinnarney for assistance. We also thank J. Gilbert for his editorial review and J. Johnson for assistance in preparation of this manuscript. This work was supported in part by NHLBI Clinical Investigator Development Award #K08 HL 03266, by Cancer Center Support CORE Grant P30 CA 21765, by the Hartwell Foundation, and by the American Lebanese Syrian Associated Charities (ALSAC).

RECEIVED 15 JULY; ACCEPTED 30 DECEMBER 1998

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